

AMENDMENTS TO THE SPECIFICATION

On page 1, insert before paragraph [0002] the following title:

BACKGROUND OF THE INVENTION

On page 2, insert before paragraph [0008] the following title:

SUMMARY OF THE INVENTION

On page 29, after paragraph [0118], replace the title as follows:

Figure Legends BRIEF DESCRIPTION OF THE DRAWINGS

On page 32, after paragraph [0134], replace the title as follows:

Examples DETAILED DESCRIPTION OF THE INVENTION

On page 28, please replace paragraph [0115] with the following amended paragraph:

[0115] Preferably, the cyclin-dependent kinase is Cdk2. Such an aptamer has a "V" sequence which is a mutant of the amino acid sequence QVWSLWALGWRWLRRYGNM (SEQ ID NO: 1), said mutant having from one to five, preferably one to three amino acid changes with respect to said sequence. A particularly preferred anti-Cdk2 aptamer has a peptide recognition domain comprising or consisting of the amino acid sequence QVWSSWALGWRWLRRYGWGM (SEQ ID NO: 2).

On page 28, please replace paragraph [0116] with the following amended paragraph:

[0116] As a preferred pro-apoptotic molecule, Bax can be cited. Anti-Bax aptamers of the invention have a peptide recognition domain comprising or consisting of a mutant of the amino acid sequence

P R G A P M W M R W V C Q M L E T M F L (SEQ ID NO: 3), said mutant having from one to five, preferably one to three amino acid changes with respect to said sequence. Preferably, the peptide recognition domain comprises or consists of the amino acid sequence

P R G A P M W L R C V C Q M L E T K F L (SEQ ID NO: 4).

On page 29, please replace paragraph [0120] with the following amended paragraph:

[0120] Fig. 1. A mutant aptamer with enhanced affinity for its target. (a) Interaction mating assay between LexA-Cdk2 and aptamer 10, two strains carrying aptamer 10M, and a non-interacting aptamer C4, using three different sensitivity LexAop-lacZ reporters. (b) Western blot assay using an anti-TrxA antibody. Diploid exconjugates were grown in galactose containing medium, and proteins were extracted and subjected to a Western blot analysis with anti-TrxA antibody. (c) Strength of interaction phenotypes as determined by fluorescence from a LexAop-GFP reporter plotted against Kds measured in evanescent wave experiments. Fluorescence values are in arbitrary units (a.u.). (d) Sequence of the variable regions of aptamers 10 (SEQ ID NO: 1) and 10M (SEQ ID NO: 2).

On page 29 and 30, please replace paragraph [0122] with the following amended paragraph:

[0122] Fig. 3. Targeted ubiquitination of LexA-Cdk2 by aptamer-*hect* fusions. (a) Design of a "modifier", inspired by the structure of *hect* domain

containing ubiquitin ligases. (b) Western blot analysis of LexA-Cdk2 (upper panel) and TrxA-*hect* or aptamer-*hect* fusions (lower panel) using anti-LexA and anti-TrxA antibodies respectively. (c) Western analysis of LexA-Cdk2 when aptamer-hect fusions are expressed by growth overnight in a medium that does or does not contain CuSO₄ and that does or does not express Myc-tagged ubiquitin. (d) Western blot analysis of LexA-Cdk2 and LexA-7Lys (SEQ ID NO: 10) -Cdk2 when TrxA-, 8- and 10M-*hect* fusions are expressed, using the anti-LexA antibody.

On page 32, please replace paragraph [0131] with the following amended paragraph:

[0131] Figures 13, 14 and 15 : construction of Aptamer dimers. Fig. 13 : Linkers of the 5-Glycine (SEQ ID NO: 5) and (4-Glycine 1 Serine) X3 (SEQ ID NO: 6) type; Fig. 14 Linkers of the HA type ; Fig. 15 : Direct fusion of the monomeric aptamers, without an intervening linker molecule.

On page 33, please replace paragraph [0139] with the following amended paragraph:

[0139] A pool of 15,000 mutants was created. To obtain a measure of the efficiency of the PCR mutagenesis, the V regions of two clones from this pool were sequenced ; sequencing revealed that these carried 3 and 4 mutations that resulted in 1 and 3 amino acid changes respectively. To identify tighter-binding variants from this pool, advantage was taken of the existence of different LexA operator reporter genes with different sensitivities. A strain that expressed a LexA-Cdk2 bait and that carried pRB1840, a relatively insensitive lacZ reporter (13) with a single synthetic LexA operator was used initially. This reporter was not activated by aptamer 10 (Fig. 1a). The pool of PCR mutagenized plasmids was introduced into this strain,

and transformants that gave rise to blue colonies were identified. The V regions of the 7 plasmids thus identified carried identical nucleotide changes, and thus were presumably not independent. The nucleotide changes resulted in two amino acid changes: Leu to Ser at V region position 5, and Asn to Gly at position 19 (Fig. 1d) :

Aptamer 10 : QVWSLWALGWRWLRRYGNM (SEQ ID NO: 1)

Aptamer 10M : QVWSSWALGWRWLRRYGWGM (SEQ ID NO: 2)

On page 34, please replace paragraph [0142] with the following amended paragraph:

[0142] The gain in affinity was measured by two different means. First, a LexAop-GFP reporter gene was used to quantify interactions between anti-Cdk2 aptamers and LexA-Cdk2. Mean fluorescence obtained from each interaction was plotted against Kds measured in evanescent wave experiments (1). This plot followed a logarithmic equation (Table 1, and Fig. 1c). This equation was used to calculate the Kd of the interaction between LexA-Cdk2 and aptamer 10M from the fluorescence it conferred (Table 1). Evanescent wave experiments were also performed with purified aptamer 10M and His6 (SEQ ID NO: 9) -Cdk2 (12). The measured Kd was 5nM (not shown), quite close to the 2nM Kd calculated from the GFP data.

On page 42 and page 43, please replace paragraph [0171] with the following amended paragraph:

[0171] *Modifiers.* DNA encoding the *hect* domain of yeast RSP5 by PCR was isolated using the oligonucleotides 5'-
ATATCTCGAGATTAAAGTACGTAGAAAGAAC-3' (SEQ ID NO: 11) and 5'-
ATATGTCGACGGATCCTCATTCTGACCAAACCCCTATG-3' (SEQ ID NO:

12) which respectively contained an XhoI site, and BamHI and SalI sites. The PCR product was subcloned into XhoI-cut pJG4-4 (ref. 42), which contains a Trp1 marker, a 2 μ replication origin, and that directed the expression of native proteins under the control of the GAL1 promoter, to create pJG4-4(*hect*). TrxA and peptide aptamers were amplified using the oligonucleotides :

5'-

GGAGGCGAATTGCCGCCACCATGGCCGATAAAATTATTCACCTGACT
GACG-3' (SEQ ID NO: 13) and

5'-ATATCTCGAGCGCCAGGTTAGCGTCGAGGAAC-3' (SEQ ID NO: 14), which contained respectively an EcoRI site followed by an initiator codon in a Kozak context, and an XhoI site. The PCR products were inserted into EcoRI/XhoI-cut pJG4-4 (*hect*). To express the *hect* domain only, the 5' oligonucleotide :

5'-

ATATGAATTGCCGCCACCATGGCCATTAAAGTACGTAGAAAGAACATT
TTTGAG-3' (SEQ ID NO: 15),

was used, which contained an EcoRI site and an initiator codon in a Kozak context with the above-described 3' oligonucleotide to PCR the *hect* domain from RSP5.

This fragment was introduced into EcoRI/XhoI-cut pJG4-4. The mutant *hect* domain was constructed using the Transformer kit from Clontech, according to the manufacturer's instructions, using the mutagenic oligonucleotide :

5'-GCCAAAATCTCACACAGCTTTAACAGAGTTG-3' (SEQ ID NO: 16)

to change the *hect* active site cysteine to alanine, and the selection oligonucleotide :

5'-CGCTAACCTGGCGCCTAGGATTAAAGTACGTAG-3' (SEQ ID NO: 17)

to change to the XhoI site on the vector to an AvrII site.

On page 43, please replace paragraph [0172] with the following amended

paragraph:

[0172] For experiments with Myc-tagged ubiquitin, the modifiers were expressed from another vector. To this end, we amplified 5-hect, 8-hect, and 10M-hect fusions described above using oligonucleotides :

5'-ATATGTCGACGGATCCTCATTCTGACCAAACCCTATG-3' (SEQ ID NO: 18)

and

5'-

GGAGGCGAATTGCCGCCACCCATGGCCGATAAAATTATTCACCTGACT
GACG-3' (SEQ ID NO: 19).

On page 43, please replace paragraph [0175] with the following amended paragraph:

[0175] *LexA-7Lys-Cdk2*. To start, the bait plasmid encoding LexA-Cdk2 (1) was used. DNA that encoded a stretch of 7 lysines was constructed by annealing the oligonucleotides :

5'-AATTGAAGAAGAAAAAAAAGAAAAAGC-3' (SEQ ID NO: 20)

and

5'-AATTGCTTTCTTTTCTTC-3' (SEQ ID NO: 21)

and this duplex was introduced into the EcoRI site of the bait plasmid. The ligation mixture was treated with EcoRI, it was introduced into *E. coli* XL-1 blue, and cells that bore plasmids containing the insert were identified by PCR.

On page 44, please replace paragraph [0178] with the following amended paragraph:

[0178] To measure interaction phenotypes with the LexAop-GFP reporter

gene, overnight liquid cultures were grown from diploid exconjugants in Ura-His-Trp- galactose liquid medium. Fluorescence was measured with a FACStar plus (Becton-Dickinson) illuminated with two argon lasers tuned to 488nm and to multiline UV. Recordings were made with a 530-/+15nm filter to measure yeast fluorescence. The FL3-2 voltage (background) was set using yeast that did not show an interaction phenotype. 30,000 cells were analyzed for each interaction, and mean fluorescence of the yeast population above background was determined using the CellQuestTM software package (Becton-Dickinson).

On page 45 and page 46, please replace paragraph [0183] with the following amended paragraph:

[0183] *Selection of the high affinity Bax aptamer B16M103:* Selection of the parental Bax aptamer B16 is described in Example 4. Aptamer B16 comprises a 20 amino-acid variable region displayed in a thioredoxin platform as previously described (ref.1). The variable region of B16 has the amino acid sequence : Variable region B16 : PRGAPMWMRWVCQMLETMFL (SEQ ID NO: 22).

On page 46, please replace paragraph [0184] with the following amended paragraph:

[0184] Mutagenic PCR was carried out on the variable region of B16 as described for Cdk2 aptamers. Amongst the obtained mutants, aptamer B16M103 was selected on the basis of high affinity and high selectivity for Bax (Figures 5, 6 and 7). The anti-Bax aptamer B16M103 (also designated “M103”) comprises a variable region having the amino acid sequence shown below, embedded in a thioredoxin platform as previously described (ref. 1) :

Variable region M103 : PRGAPMWLRCVCQMLETKFL (SEQ ID NO: 23)

On page 46 and page 47, please replace paragraph [0187] with the following amended paragraph:

[0187] *Dimers with a 5 Gly or a (4Gly-1Ser)x3 linker* : DNA encoding the aptamer destined to position 1 was obtained as described above. DNA encoding the aptamer destined to position 2 was PCR amplified using the oligonucleotides olApt2 and olTrxend2. The PCR product was digested with NcoI and XhoI. DNA encoding the 5 Gly linker was obtained by hybridizing the oligonucleotides olPoly5Gly and olPoly5Glyrev. DNA encoding the (4Gly-1Ser)x3 linker was obtained by hybridizing the oligonucleotides olGly4Ser and olGly4Serrev. The resulting duplexes contained a NheI cohesive end on their 5' terminus and a NcoI cohesive end on their 3' terminus. One-step ligations of EcoRI/XhoI-cut pJG4-5, both digested PCR products, and either one of the duplexes encoding the linkers were performed (See Figure 13)

Oligonucleotide sequences :

OlHAaptsens : 5' CGGGGTACCTTGGGT CCTACCCTTATGATGTG 3' (SEQ ID NO: 24)

olTrxEnd1 : 5' ATTTAAGCTAGCGGCCAGGTTAGCGTCGAGGAAC 3' (SEQ ID NO: 25)

olNoLink : 5' TTAATAGCTAGCATGAGCGATAAAATTATTCACC 3' (SEQ ID NO: 26)

olTrxEnd2 : 5' AATATCTCGAGCTAGGCCAGGTTAGCGTCGAGG 3' (SEQ ID NO: 27)

olLinkHA : 5' TTAATAGCTAGCTTGGGT CCTACCCTTATGATGTG 3' (SEQ ID NO: 28)

olApta2 : 5' TTATTCCATGGTATGAGCGATAAAATTATTCAAC 3' (SEQ ID NO: 29)

olPoly5Gly: 5' CTAGCGGTGGTGGTGGCGGC 3' (SEQ ID NO: 30)

olPoly5Glyrev : 5' CATGCCGCCACCACCCACCG 3' (SEQ ID NO: 31)

olGly4Ser: 5'

CTAGCGGTGGTGGTGGCTCCGGTGGTGGCTCCGGTGGTGGCTC
C 3' (SEQ ID NO: 32)

olGly4Serrev:

5'CATGGGAGGCCACCACCGGAGGCCACCACCGGAGGCCACCAC
CG (SEQ ID NO: 33)

After the claims, commencing on a separate page, insert at the end of the specification the following Abstract:

ABSTRACT OF THE DISCLOSURE

Disclosed herein are intracellular recognition molecules and their use,
for example, for targeted modification of intracellular compounds.